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Detection of *p53* and K-*ras* mutations in sputum of individuals exposed to smoky coal emissions in Xuan Wei County, China

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Lung cancer mortality rates in the Xuan Wei County population are among the highest in China and are associated with exposure to indoor emissions from the burning of smoky coal. Previous studies of lung tumors from both non-smoking women and smoking men in this region showed high frequencies of mutations, consisting mostly of G→T transversions in the *p53* tumor suppressor gene and K-*ras* oncogene, suggesting that these mutations were caused primarily by polycyclic aromatic hydrocarbons. In this study sputum samples from 92 individuals with no evidence of lung cancer from Xuan Wei County were screened for *p53* and K-*ras* mutations. Sputum cells were collected on glass slides by sputum cytocentrifugation, stained and cytopathologically analyzed. Cytologically non-malignant epithelial cells were taken from each sputum sample using a laser capture microdissection microscope and molecularly analyzed. Cells taken from the sputum of 15 (16.3%) individuals were mutation positive, including 13 (14.1%) individuals each with a *p53* mutation, 1 (1.1%) individual with a K-*ras* mutation and 1 (1.1%) individual with a *p53* and a K-*ras* mutation. *p53* mutations were found in both the sputum of individuals with evidence of chronic bronchitis (3 of 46 or 6.5%) and those without evidence of this disease (11 of 46 or 23.9%). Therefore, mutations in the *p53* gene and, to a lesser extent, the K-*ras* gene were frequent in non-malignant epithelial cells taken from the sputum of individuals without evidence of lung cancer who were exposed to smoky coal emissions in Xuan Wei County and were at a high risk for developing the disease.

Abbreviations: DGGE, denaturing gradient gel electrophoresis; LOH, loss of heterozygosity; PAHs, polycyclic aromatic hydrocarbons; SSCP, single-stranded conformational polymorphism; XWC, Xuan Wei County.

Introduction

Lung cancer remains the most common cause of death from cancer world wide, with tobacco smoke being the primary cause (1,2). In Xuan Wei County (XWC), Yunnan Province, lung cancer rates per 100 000 from 1973 to 1979 were 25.3 for women and 27.7 for men, which represent eight times and four times the Chinese national average rates for women and men, respectively. These data were unusual since few women smoked and most men smoked (3). Several studies have provided strong support for the high lung cancer rates in the population in XWC being associated with the use of smoky coal, a low sulfur, medium volatility bituminous coal, for cooking and heating in homes without chimneys (4–10). These emissions contained primarily organic matter, including a high proportion of polycyclic aromatic hydrocarbons (PAHs) (5,7). Both non-smokers, mostly women, and smokers, mostly men, who were exposed to these emissions showed high levels of PAH metabolites and benzo[*a*]pyrene adducted guanines in their urine (4,5,7,9). An organic extract of smoky coal emissions was mutagenic in *Salmonella* (11) and also tumorigenic, even more so than cigarette smoke extracts, in a murine skin tumor assay, presumably due to a higher concentration of PAHs in the smoky coal emissions (12). Taken together, these data suggest an association between exposure to smoky coal emissions and increased lung cancer risk and have led to a strong interest in developing biomarkers for the diagnosis and early detection of lung cancer among this unique population of XWC.

Our previous studies of lung tumors obtained from lung cancer patients from XWC showed high frequencies of mutations in both the *p53* tumor suppressor gene and K-*ras* oncogene (13,14). In particular, the predominance of G→T transversions in the tumors of both non-smoking women and smoking men indicated that these mutations were caused primarily by exposure to PAHs present at high proportions in smoky coal emissions (12–14). In this study we have hypothesized that these gene mutations may occur early in individuals who have a history of long-term exposure to smoky coal emissions in XWC and may be detected in non-invasive specimens, such as sputum, of this unique population, using sensitive methods.

Previously we developed a method that combines sputum cytology and laser capture microdissection techniques to isolate tumor cells from the sputum of lung cancer patients for analysis of *p53* and K-*ras* mutations. We have demonstrated that this method allowed detection of low fraction mutations occurring throughout the exons of the *p53* gene as compared with available sensitive methods that allowed detection of mutations within only a few specific codons in DNA extracted

directly from mixed cell types in sputum (15). Here we apply this method to screen for *p53* and K-*ras* mutations in cytologically non-malignant epithelial cells taken from the sputum of 92 individuals who had no evidence of lung cancer but were exposed to smoky coal emissions in XWC and considered to be at high risk for developing lung cancer.

Materials and methods

Subjects and sputum samples

Individuals who donated the sputum samples analyzed in this study were part of a previous study (8). These individuals were examined on a minimum of clinical symptoms and chest X-ray analysis at Xuan Wei Hospital. They were found to have no evidence of lung cancer. Each individual who provided informed consent to participate in this study also answered a standardized closed questionnaire on demographic information, smoking history and familial and personal medical history, as well as information on other variables (8). To protect the human subjects, this study was conducted according to the recommendations of the World Medical Association *Helsinki Declaration* (1989) (16). The research protocol met the requirements for protection of human subject certification by the US EPA.

Sputum samples were obtained from a total of 92 individuals, including 28 women (26 non-smokers and 2 smokers) with an average age of 56.3 (33–78) years and 64 men (3 non-smokers and 61 smokers) with an average age of 57.6 (23–80) years. Of these individuals, 46 had symptoms of chronic bronchitis, with excessive bronchial mucus and a chronic cough for 3 months or more in at least three consecutive years and without any other disease that could account for these symptoms, and 46 had no such symptoms. For each individual, sputum samples were collected first thing in the morning on five consecutive mornings. Each subject was instructed to rinse his/her mouth with water to remove extraneous material, to take a deep breath and cough deeply and expectorate into a plastic cup. Each morning sputum sample was stored in 40 ml of Saccomanno's fluid (39% ethanol, 3% polyoxyethylene and 2% isopropanol; Lerner Laboratories, Pittsburgh, PA) to fix and preserve the cells. The sputum samples were stored at 4°C and transported to the USA by air. To collect cells, each sputum sample in Saccomanno's fluid was blended for 8–15 s in a blender to break the mucus and free the cells. The sample was then centrifuged at 600 *g* for 10 min. The supernatant was discarded and the cell pellet was resuspended in fresh Saccomanno's fluid by vortexing to achieve a cell concentration of ~10^6 cells/ml. The cells were subjected to cytological examination using the method described by Saccomanno in order to determine whether the sputum samples were derived from the lower respiratory tract and also to confirm the presence of bronchial epithelial cells (8).

Isolation of epithelial cells from sputum

An aliquot of each sputum sample containing the equivalent of 5000 cells was centrifuged and the Saccomanno's fluid discarded. The cells were resuspended in 1 ml of phosphate-buffered saline and transferred to a Cyto-Tek specimen Chamber. The chamber was then fixed onto a holder equipped with a membrane filter (VWR, Bridgeport, NJ) and a glass slide and centrifuged using a Cyto-Tek centrifuge (VWR). The slide was recovered from the chamber and air dried. The cells retained on the filter were stained with eosin and hematoxylin and were cytologically examined. About 3–10% of the cells in each sputum aliquot were bronchial epithelial cells in a background of other cell types, consisting mostly of leukocytes and buccal epithelial cells (referred to in this study as buccal cells). The sputum of individuals with chronic bronchitis had a large number of inflammatory cells, consisting mostly of neutrophils. Approximately 150, morphologically benign, epithelial cells were captured on a 'cap', using a laser capture microdissection microscope. Buccal cells were also taken separately, molecularly analyzed and compared with matched bronchial epithelial cells.

DNA extraction and mutation analysis

Each captured cell sample was lyzed by adding 15 μl of lysis solution (40 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.5% Tween-20 and 0.5 μg/μl proteinase K) directly to each cap. The resulting cell lysate was recovered in a microcentrifuge tube by a quick spin of the tube then heated at 95°C for 5 min to inactivate the proteinase K (17).

Analysis of mutations in the K-*ras* and *p53* genes was carried out as described previously (17,18). Briefly, for K-*ras* mutation analysis an aliquot of each cell lysate containing the equivalent of 20 bronchial epithelial cells was used for PCR in a 25 μl reaction mixture containing reagents and [α-^{32}P]dATP (NEN, Boston, MA). The PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) and mutant sequences were isolated and further characterized for mutations (17,18). For analysis of mutations in the *p53* gene the equivalent 20 cells from each cell lysate were used to amplify each of the three fragments corresponding to exons 5–6, 7 and 8 separately in a 25 μl reaction mixture containing reagents and [α-^{32}P]dATP and an appropriate pair of primers. The PCR products were analyzed by single-stranded conformational polymorphism (SSCP) (18). Bands appearing in each autoradiogram corresponding to a mutant allele(s) and those corresponding to the wild-type allele in each cell sample were scanned, using a densitometer (UMAX Powerlook 1120), and the intensity of each band was measured, using NIH Scion software. The fraction of mutant allele(s) was then estimated as the ratio between the intensity of the mutant allele(s) and the sum of the intensities found for the mutant allele(s) and the wild-type allele in each cell sample. Mutant alleles appearing on the gel were isolated, further amplified and characterized by sequencing, using an ABI PRISM 377 automatic sequencer.

Results

Mutation analysis

In our preliminary experiments we extracted genomic DNA from an aliquot of each sputum sample and analyzed it for K-*ras* mutations, using PCR and DGGE, and for *p53* mutations, using PCR and SSCP, which can detect mutations present at mutant fractions of 5 and 10% in a wild-type background, respectively (17,18). Using these methods, we were unable to detect any mutation in these sputum samples. Subsequently, an aliquot of DNA from 46 individuals was further analyzed using a more laborious but sensitive method that involved repeated steps of PCR and restriction enzyme digestion to enrich for mutations occurring at a few specific codons of these genes (15). This method allowed us to detect a mutation in codon 248 of the *p53* gene and another mutation in codon 12 of the K-*ras* gene in sputum DNA of one individual (subject B16 in Table I). These results suggest that K-*ras* and *p53* mutations were present in only a small fraction of sputum cells of individuals exposed to smoky coal emissions in XWC. Such low fraction mutations may also occur in many other codons of the *p53* gene, but it would be impractical to screen for them using existing sensitive but codon-specific methods. In order to facilitate the detection of low fraction mutations that may occur in the *p53* and K-*ras* genes in sputum we applied a method combining cell cytocentrifugation and a laser capture microdissection microscope to isolate only bronchial epithelial cells from the large excess of other cell types present in each sputum sample. The isolated cells were then analyzed for *p53* mutations, using PCR and SSCP, and for K-*ras* mutations, using PCR and DGGE. We have previously

Table I. Summary of subjects and mutations in the K-*ras* and *p53* genes

Subject	K-*ras* mutations	*p53* mutations
M002		E7 codon 242 TGC→TTC
M005		E7 codon 242 TGC→TTC
M008		E8 codon 269 AGC→AAC
M010		E7 codon 248 CGG→CTG
M026		E7 codon 248 CGG→CTG
M028		E7 codon 258 GAA→TAA
M042		E7 codon 248 CGG→CTG
M058		E7 codon 258 GAA→TAA
M060		E5 codon 139 AAG→TAG
M069		E7cod.245 GGC→GTC
M075		E8 codon 282 CGG→CAG
B16	Codon 12 GGT→GTT (Gly→Val)	E7 codon 258 GAA→TAA
B42		E7 codon 248 CGG→CTG
B47	Codon 13 GGC→GCC (Gly→Ala)	
B48		E5 codon 156 CGC→CCC

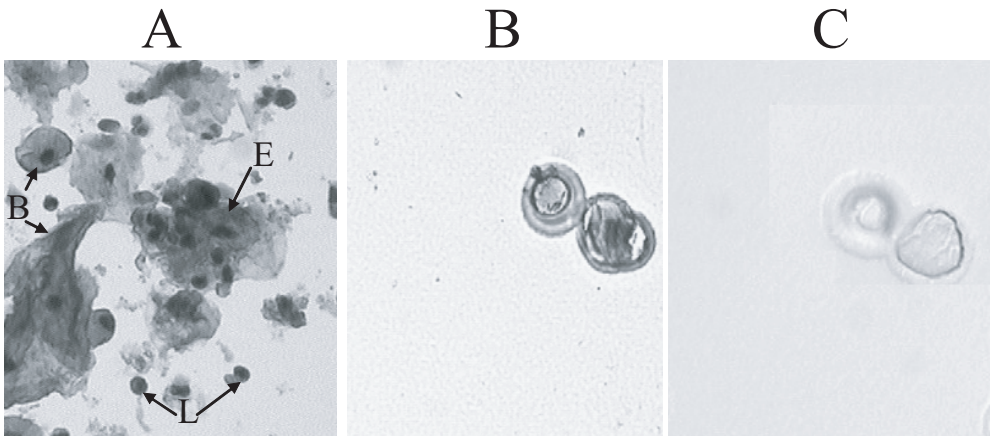

Fig. 1. An example of histopathological analysis of a sputum sample of one individual with evidence of chronic bronchitis. (**A**) A group of cells were collected on a glass slide by cytocentrifugation and consisted of a mixture of mostly leucocytes (L), bronchial epithelial cells (E) and buccal epithelial cells (B) (magnification ×100). (**B**) The cells 'captured' on a 'cap' using a laser capture microdissection microscope. C, the 'cap' after digestion with proteinase K; the cellular content was totally lyzed, leaving behind empty spaces.

demonstrated that this approach allowed us to detect *p53* and K-*ras* mutations present in a few tumor cells isolated from a large excess of other cell types in sputum of lung cancer patients from XWC (15). We here applied this same approach to analyze these same gene mutations in sputum of individuals without evidence of lung cancer who were exposed to smoky coal emissions in XWC.

Figure 1A shows the results of a cytological examination of cells collected on a slide by cytocentrifugation of a sputum sample obtained from one of the subjects. These cells consisted of a mixture of mostly leukocytes (L) and buccal cells (B) and 3–10% morphologically benign bronchial epithelial cells. In the sputum of individuals with chronic bronchitis, inflammatory cells, mostly neutrophils, were abundant. Bronchial epithelial cells present in aliquots taken from the sputum of individuals without evidence of chronic bronchitis all appeared normal looking while a few of those present in the aliquots of sputum of individuals with evidence of the disease appeared atypical. There were no morphologically malignant cells present in any of the aliquots taken from the 92 sputum samples. About 150 epithelial cells were laser captured on each 'cap'. Cell samples taken from the sputum of individuals with evidence of chronic bronchitis included both normal looking epithelial cells and those appearing morphologically atypical (Figure 1A). The captured cells shown in Figure 1B were lysed as shown in Figure 1C and an aliquot of each cell lysate was used for mutation analysis.

Figure 2 shows an example of the molecular analysis of mutations in codons 12 and 13 of the K-*ras* gene by DGGE (Figure 2A) and *p53* mutations by SSCP (Figure 2B) in cells taken from the sputum of five subjects (subjects B47, B48, B16, B42 and M002). In Figure 2A, for subject B47, a pattern of K-*ras* mutant sequences (each indicated by an arrowhead) appeared in addition to the wild-type allele (wt) and a background of polymerase-induced sequences. This mutant corresponded to a GGC→GCC mutation occurring in codon 13 of the K-*ras* gene and was only detected in bronchial epithelial cells (ep) and not in matched buccal cells (bu). Subject B48 showed no detectable mutant sequences in either bronchial epithelial cells or matched buccal cells. In Figure 2B, SSCP analysis of the *p53* gene showed that subjects B16, B42 and

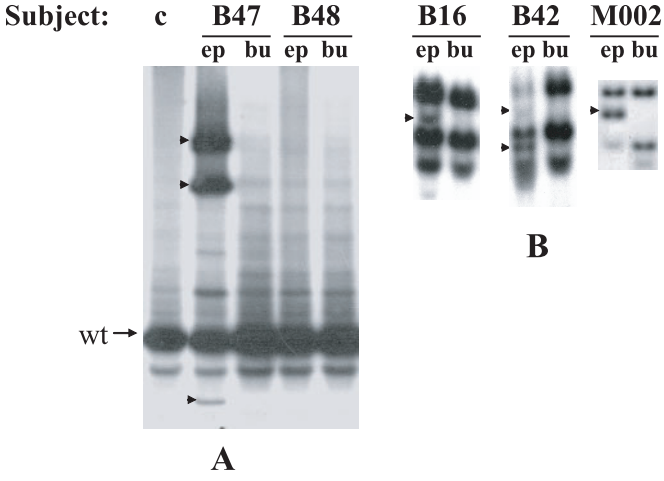

Fig. 2. *p53* and K-*ras* mutation analysis. The figure shows an example of K-*ras* mutation analysis by PCR + DGGE (**A**) and *p53* mutation analysis by PCR + SSCP (**B**) in non-malignant epithelial cells (lane ep) and matched buccal cells (lane bu) taken from the sputum of five of the subjects shown in Table I. In (A) DGGE analysis showed that subject B47 revealed mutant sequences (indicated by arrows) corresponding to a K-*ras* codon 13 GGC→GCC mutation, in addition to the wild-type sequence (wt) and minor polymerase-induced sequences. This mutation was only detected in the bronchial epithelial cells (lane ep) and not in the matched buccal cells (lane bu). There were no mutant sequences in the cell samples taken from subject B48, or the genomic DNA used as a negative control (lane c). In (B) aliquots of cell samples taken from three additional subjects were analyzed by PCR + SSCP for *p53* mutations. Subjects B16, B42 and M002 each showed mutant alleles in epithelial cells (lanes ep), corresponding to a GAA→TAA at codon 258, a CGG→CTG at codon 248 and TGC→TTC at codon 242 of the *p53* gene, respectively. These mutant alleles were not detected in the matched buccal cells (lanes bu).

M002 each showed mutant sequences that were found to correspond to a transversion, including GAA→TAA at codon 258, CGG→CTG at codon 248 and TAC→TTC at codon 242, respectively. In all these subjects the mutations were only detected in epithelial cells (lanes ep) and not in matched buccal cells (lanes bu).

Table I summarizes the *p53* and K-*ras* mutations identified in epithelial cells taken from sputum of 15 of the 92 individuals,

Table II. *p53* and K-*ras* mutations in sputum epithelial cells according to gender and smoking status of the subjects

Subjects	Smoking status	Mutations	
		p53	K-*ras*
28 females (30.4%)	26 non-smokers	2	0
	2 smokers	1	0
64 males (69.6%)	3 non-smokers	0	0
	61 smokers	11	2
Total 92		14 (15.2%)[a]	2 (2.2%)[a]

[a]One subject had both a *p53* and a K-*ras* mutation.

including one individual with a K-*ras* mutation (subject B47), one individual with a K-*ras* mutation and a *p53* mutation (subject B16) and 13 individuals each with a *p53* mutation. The 14 *p53* mutations included 10 (71.4%) G→T, 1 (7.1%) G→C and 1 (7.1%) A→T transversion and 2 (14.3%) G→A transitions. As shown in Table II, three (10.7%) *p53* mutations were found among the 28 women, including 2 mutations among the 26 non-smokers and 1 mutation in the two smokers. No K-*ras* mutations were found among these women. Eleven (17.2%) *p53* mutations and two K-*ras* mutations were found among the 64 men, all among the 61 smokers.

The two K-*ras* mutations were detected at a mutant fraction of ~25 and 30% in cells taken from subjects B16 and B47 (Figure 2A and Table I), respectively. On an individual basis, *p53* mutations were detected among the14 subjects at an average mutant fraction of 25%, ranging from 15 (the codon 258 GAA→TAA mutation in subject B16) to 60% (the codon 242 TGC→TTG mutation in subject M002) (see Figure 2B and Table I).

Discussion

The results of this study demonstrate that *p53* and, to a lesser extent, K-*ras* mutations were present in non-malignant bronchial epithelial cells taken from the sputum of individuals without evidence of lung cancer who were exposed to smoky coal emissions in XWC. These results are consistent with those of our previous studies of lung tumors obtained from lung cancer patients from XWC showing a particularly high proportion of G→T transversions (13,14), suggesting that these mutations were caused primarily by PAHs. Our results also showed that the *p53* gene was more frequently mutated than the K-*ras* gene in sputum of these individuals (15.2 versus 2.2%, Table II). This is in line with the results of our previous studies showing that the frequency of *p53* mutations was higher than that of K-*ras* mutations in lung tumors of lung cancer patients from XWC (13,14). Furthermore, the average fraction of these mutations in bronchial epithelial cells was 25% among the 14 individuals showing a *p53* mutation and 28% among the 2 individuals showing a K-*ras* mutation, suggesting that only a small fraction of the epithelial cells taken from the mutation-positive sputum samples contained the mutation. Since bronchial epithelial cells accounted for only 3–10% of all cell types in the sputum of these individuals, the average fraction of mutations in the *p53* and K-*ras* genes among all sputum cell types would be between 0.75 and 2.8%. These mutations were only present in bronchial epithelial cells and were not detected in matched buccal epithelial cells taken from the same mutation-positive sputum samples or from known negative control DNA samples that did not contain the mutations analyzed in parallel in the same experiments (data not shown). Therefore, it is unlikely that these mutations were generated by DNA polymerase-induced errors or by cross-contamination.

The *p53* mutations were mostly found in the sputum of smoking men (11 of 61 or 18.0%) and also, but to a lesser extent, in those of non-smoking women (2 of 26 or 7.7%) (Table II). Due to the small number of samples analyzed and/or mutations found, it is unclear whether the higher *p53* mutation frequency found in smokers, compared with non-smokers, might be due to gender difference and/or a cumulative effect of PAHs from both smoky coal emissions and cigarette smoke among smokers (19).

Long-term exposure to tobacco smoke and smoky coal emissions has been suggested to be a risk factor for respiratory diseases, including chronic bronchitis (19). Among the 92 individuals investigated in this study, 46 showed clinical evidence of chronic bronchitis and the remaining 46 had no evidence of the disease. *p53* mutations were found in 6.5% (3 of 46) of the individuals with the disease and in 23.9% (11 of 46) of the individuals without the disease. However, because of the small number of subjects analyzed, it is unclear whether chronic bronchitis may have any effect on *p53* or K-*ras* mutation frequencies or mutant fractions in the sputum of individuals exposed to coal combustion and tobacco smoke in XWC.

It is not known whether the presence of *p53* and K-*ras* mutations in morphologically benign bronchial epithelial cells in sputum was associated with an increased risk of lung cancer development among individuals exposed to smoky coal emissions in XWC. A few studies have investigated these gene mutations in DNA extracted from sputum using sensitive methods. K-*ras* mutations have been detected in the sputum of lung cancer patients (20–22) and detection of these mutations in sputum correlated with the presence of identical mutations in matched lung tumors (21), suggesting that detection of these mutations in sputum may be a useful biomarker for lung cancer. K-*ras* mutations have also been found in the sputum of individuals without lung cancer but who had evidence of bronchitis, asthma and pneumonia (22). They were also found in bronchoalveolar lavage of former lung cancer patients who no longer had evidence of the disease but were at high risk of developing a second primary lung cancer (23). Taken together, these results suggest that K-*ras* mutations occurred early and may be useful markers for early lung cancer diagnosis.

There have been fewer studies of *p53* mutations in sputum samples. This is primarily due to the fact that, unlike K-*ras* mutations that have been found primarily in codon 12 and, to a much lesser extent, in codons 13 and 61 of the K-*ras* gene (17), *p53* mutations have been found at >100 sites in the *p53* gene in lung and other tumor types (24). Furthermore, their occurrence in only a small fraction of sputum cells made it impractical to determine a full *p53* mutational spectrum by analyzing DNA extracted directly from whole sputum cells. Nevertheless, previously Mao *et al*. (25) performed a retrospective study of mutations in stored sputum samples obtained from individuals prior to clinical diagnosis, using a highly sensitive PCR–oligohybridization method. Of 15 patients who later developed lung cancer, 10 contained a K-*ras* or *p53* gene mutation in their lung tumors. In 8 of these 10 patients an

identical mutation to that found in the primary lung tumor, including 7 K-*ras* mutations and 1 *p53* mutation, was detected in at least one sputum sample. The earliest detection of a clonal population of cancer cells in sputum was in a sample obtained 1 year prior to clinical diagnosis.

It is unclear from our study whether the detection of mutations in sputum was associated with the presence of identical mutations in the bronchial epithelium of these individuals because we did not have matched bronchial tissues from any of the individuals in this study. Furthermore, we did not have sputum of individuals who were not exposed to smoky coal emissions available for use as controls in this study. Nevertheless, *p53* mutations were found in previous studies in abnormal and/or histologically normal bronchial epithelium of patients with lung cancer and in those of smokers and former smokers who had no evidence of lung cancer. For instance, loss of heterozygosity (LOH) at the *p53* gene locus and overexpression of p53 protein have been reported in the dysplastic bronchial epithelium of patients without lung cancer (26). A study of a smoker who had dysplastic changes in the bronchial epithelium without overt carcinoma revealed the widespread presence of a single somatic *p53* mutation in the bronchi, suggesting that a single progenitor bronchial epithelial clone may expand to populate broad areas of the bronchial mucosa (27). Miozzo *et al*. (28) compared the presence of clonal variations at microsatellite polymorphisms between tumor, histopathologically normal bronchial mucosa and sputum of lung cancer patients. In five cases that had sputum samples available, identical alterations were found in both the sputum and in the matched tumor and/or histologically normal bronchial mucosa, suggesting a link between the detection of microsatellite alterations in sputum and the presence of identical alterations in the matched bronchial and/or tumor tissues. Furthermore, other studies showed that LOH and microsatellite alterations were found in bronchial specimens of smokers and former smokers (29,30). Among the smokers, about half of the histologically normal biopsy specimens showed LOH, compared with the absence of such genetic alterations in specimens from non-smokers (30). Some of the alterations were found at chromosomal sites containing putative tumor suppressor genes, including at 17p13, which contains the *p53* tumor suppressor gene, in histologically normal or minimally altered bronchial epithelium of current and former smokers (29,30). Taken together, these results suggest that the bronchial epithelium of smokers and former smokers was damaged by tobacco smoke mutagens. It may be that the respiratory epithelium of XWC residents was heavily damaged by mutagens, such as PAHs, found in both smoky coal emissions and tobacco smoke. Therefore, it may not be unexpected that in our study *p53* and K-*ras* mutations were found in the sputum of 16% of the individuals investigated who were exposed to smoky coal emissions and most of whom were also smokers.

The subjects who participated in this study were anonymous and were not followed up further. Therefore, the relationship between the presence of *p53*/K-*ras* mutations in sputum and the role of these mutations in lung cancer development in these individuals is unknown. Further studies involving a larger sample size of both unexposed individuals and individuals exposed to smoky coal emissions would be required to assess the value of these mutations in sputum of XWC residents as biomarkers for early lung cancer detection.

In summary, we have demonstrated that *p53* and K-*ras* mutations are present in non-malignant bronchial epithelial cells taken from the sputum of individuals who were exposed to smoky coal emissions but had no evidence of lung cancer in XWC, suggesting that these mutations occurred early and may be associated with exposure to PAHs present in both smoky coal emissions and tobacco smoke. The prognostic value of these mutations as potentially useful biomarkers for early lung cancer detection should be evaluated by future studies involving a large number of both unexposed individuals and individuals exposed to smoky coal emissions, with long-term follow up. The combination of sputum cytocentrifugation/laser capture microdissection microscope and mutation analysis may provide a useful method for analysis of mutations in these and other genes in sputum of a large number of subjects who are at high risk of developing lung cancer.

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